REMARKS

STATUS OF THE CLAIMS

Claims 1-20 are pending. Claims 1, 2, 11, and 13 have been amended. Support for the claim amendments can be found in the originally filed specification, for example, at page 5, second full paragraph. No new matter has been added.

REJECTION UNDER 35 U.S.C. § 102(b)

The Examiner maintains the rejection of claims 1-7 and 9-12 under 35 U.S.C. § 102(b) over Furuta *et al.*; *Jpn. J. Cancer Chemother.*, 18(3):393-402, 1991 (hereinafter "Furuta"). The Examiner argues that Furuta discloses Applicant's composition "wherein said composition provides a synergistic effect in the treatment of tumors." Office Action at 2. In order to anticipate a claim, a reference must contain all elements of the claim. *See Hybritech v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379, 231 U.S.P.Q. 81, 90 (Fed. Cir. 1986). Applicant respectfully traverses the rejection and submits that Furuta does not anticipate the claimed invention.

A. Synergy

The Examiner addresses Applicant's arguments relating to the claim construction of synergy on pages 6-8 of the present Office Action. The Examiner's anticipation and obviousness rejections are based on the ordinary meaning of the term "synergy" as determined by the dictionary definitions provided by the Examiner. The Examiner continues to ignore the specific definition of therapeutic synergy in the specification and therefore continues to improperly determine the meaning of the terms used in the claims. In essence, the Examiner appears to be forcing his claim construction on the Applicant as opposed to letting the Applicant draft the claims in order to particularly

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point out and distinctly claim the subject matter which she considers to be her invention.

In examining the claims, the Examiner "must rely on the applicant's disclosure to properly determine the meaning of terms used in the claims." M.P.E.P. § 2106 relying on *Markman v. Westview Instruments*, 52. F.3d 967, 980 (Fed. Cir.) (*en banc), aff'd*, 116 S. Ct. 1384 (1996). The words in a claim may be given their ordinary meaning if the applicant has not provided a clear definition for the word in the specification. If applicant does provide a clear definition in the specification then the words in a claim are no longer given their plain meaning. M.P.E.P. § 2111.01 citing *In re Zletz*, 893 F.2d 319, 321 (Fed. Cir. 1989). In fact, when the specification provides definitions for terms appearing in the claims, the specification can be used in interpreting claim language. *Id.*, citing *In re Vogel*, 422 F.2d 438, 441 (C.C.P.A. 1970).

As the Examiner is aware, an Applicant may be her own lexicographer and define in the claims what is regarded as the invention essentially in whatever terms chosen so long as the terms are not used in ways that are contrary to accepted meanings in the art. M.P.E.P. §§ 2111.01 and 2173.01. An Applicant does not need to use the terminology used in the prior art so long as the terms used to define the invention are clear and precise. M.P.E.P. § 2173.05(a). "When the specification states the meaning a term in the claim is intended to have, the claim is examined using that meaning." *Id.* (emphasis added), citing *In re Zletz.* In fact, when an explicit definition is provided by the applicant for a term, that definition will control interpretation of the terms as it is used in the claim. M.P.E.P. § 2106 citing *Toro Co. v. White Consolidated Industries, Inc.*, 199 F.3d 1295, 1301 (Fed. Cir. 1999). The Examiner should determine if the original disclosure provides a definition consistent with any assertions made by applicant. *Id.*

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The Examiner argues that Applicant has not clearly set forth a definition for "synergy," but that the specification provides a definition for "therapeutic synergy." Office Action at 6. The Examiner also argues that the word therapeutic with synergistic or synergy is not used in all of the claims. *Id.*

Solely in an effort to expedite prosecution, Applicant has amended the independent claims to recite a "therapeutic synergistic effect." Applicant is presently claiming a therapeutic synergistic pharmaceutical composition that provides a therapeutic synergistic effect in the treatment of solid tumors. Applicant has defined a therapeutic synergistic effect as a combination of two constituents wherein the combination provides a therapeutic effect that is superior to one or the other of the constituents when used at its optimum dose (*i.e.*, maximum tolerated dose or highest non-toxic dose (HNTD)). Present application at page 5, second full paragraph. This definition can also be found in an art recognized journal article (the Corbett article cited in the specification) and is therefore not contrary to accepted meanings in the art.

Applicant has consistently used the clear definition for "therapeutic synergy," as discussed above, throughout the specification and examples further emphasizing that this definition is relied upon in the presently claimed invention. For example, the specification at page 6, second paragraph, discloses that "the maximum tolerated dose of the CPT-11/doxorubicin combination is therapeutically superior to the maximum tolerated dose of either CPT-11 or doxorubicin alone." Moreover, the combination of CPT-11 and doxorubicin produced "a therapeutic response [that] was better than for the agents alone." *Id.* at page 9, Table III, and last full paragraph; *see also*, Table IV on pages 11-12; the first full paragraph on page 13; and page 15, first paragraph.

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Applicant has provided a clear definition in the specification for the term "therapeutic synergy" and thus the plain meaning of this term should not be used. The definition of this term is not contrary to accepted meanings in the art as evidenced by the Corbett journal article cited in the specification. Because Applicant has provided an explicit definition for "therapeutic synergy," this definition will control the interpretation of the term as it is used in the claims, i.e., the claims must be examined using this definition.

The Examiner attempts to rely on *Abbott Laboratories v. Syntron Bioresearch, Inc.*, to support his position that the term "synergy" has not been defined with "reasonable clarity, deliberateness, and precision necessary for departure from [the] ordinary meaning." *Abbott Laboratories v. Syntron Bioresearch, Inc.*, 334 F.3d 1343 (Fed. Cir. 2003); Office Action at 7. In *Abbott*, the specification provided two alternative definitions for analyte. *Abbott*, at 1354-55. The court recognized that a patentee may be his own lexicographer (relying on *In re Renishaw*, 158 F.3d 1243 (Fed. Cir. 1998), so long as the term appears with "reasonable clarity, deliberateness, and precision." *Id.* at 1354 citing *In re Paulsen,* 30 F.3d 1475, 1480 (Fed. Cir. 1994). The court held that because two conflicting definitions were provided in the specification for the term "analyte," it therefore did not appear with reasonable clarity, deliberateness, and precision. Based on this rationale, the court held that the ordinary meaning of the term "analyte" should be used.

The present case is different from *Abbott* because the present specification does not provide, nor does the Examiner assert as much, conflicting definitions for the term "therapeutic synergy." In addition, as previously argued, Applicant has overcome the heavy presumption that the term "synergy" (or "therapeutic synergy") should be given its

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ordinary and customary meaning, such as the two dictionary definitions previously relied upon by the Examiner. Applicant has acted as her own lexicographer as previously argued in the Request for Reconsideration After Final Rejection filed May 21, 2003. (*See also Abbott Laboratories v. Novopharm Ltd.*, 323 F.3d 1324 (Fed. Cir. 2003) (holding that patentee had overcome the presumption that a claim term is given its ordinary and customary meaning because patentee acted as his own lexicographer by providing a definition in the specification).)

Moreover, Applicant has presented arguments, such as those below, distinguishing the claimed invention over the cited art, Furuta, by relying on the definition of synergy (or therapeutic synergy) as defined in the present specification. In essence, Applicant has overcome the heavy presumption that a claim term should be given its ordinary meaning.

Therefore, as argued in previous responses, the Examiner must examine the claims using the definition provided in the specification on page 5, and not the ordinary meaning of the word "synergy" (or "therapeutic synergy") such as those provided in the dictionaries relied upon by the Examiner. The M.P.E.P. and case law discussed above dictate that the Examiner must use that construction when applying art to the claims. The Examiner's continued failure to follow the M.P.E.P. and the case law is an egregious error.

In view of the definition of "therapeutic synergy," the cited art does not anticipate and would not have rendered obvious the claimed invention.

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B. Furuta Does Not Anticipate the Claimed Invention

Although Furuta states that treatment with CPT-11 and adriamycin (*i.e.*, doxorubicin) provides synergistic effects, this reference does not, in fact, disclose a

therapeutic synergistic effect as defined in the present specification and presently claimed.

When using one constituent, such as CPT-11, one of ordinary skill in the art would expect that the best result would be achieved at the HNTD for that constituent. However, it may be beneficial to a patient to use a smaller dose, possibly, for example, to limit side effects. A therapeutic synergistic combination allows a patient to receive a smaller dose (*i.e.*, less than the HNTD) of one or both constituents and provides results greater than would have been achieved with the HNTD if each constituent had been used individually.

Applicant determined the HNTD for CPT-11 by intravenous (i.v.) and oral routes on various tumors.[1] *See* page 7, Table I. The HNTD for CPT-11 per os (p.o.) was found to be 806.4 mg/kg (100.8 x 2 times per day x 4 days (6-9)) on PO3 tumors in B6D2F1 mice. *See* page 11, Table IV.

Additionally, Applicant determined the HNTD for doxorubicin by i.v. on PO3 tumors to be 12.4 mg/kg (6.2 x 2 days of administration). *See* page 11, Table IV. This result is also consistent with what is known in the art. *See*, for example, Kolfschoten, G. M., *et al.*, Development of a Panel of 15 Human Ovarian Cancer Xenografts for Drug Screening and Determination of the Role of the Glutathione Detoxification System,

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1 In the clinic, CPT-11 is generally administered by i.v. and oral routes. The HNTD for CPT-11 by i.v was found to be 346.2 mg/kg in PO3 tumors on B6D2F1 mice. *See* page 7, Table 1. This HNTD is consistent with what is known in the art. *See*, for example, Chatelut, G. S., *et al.*, Comparison of Intraperitoneal and Intravenous Administration of Irinotecan (CPT-1) in a Murine Peritoneal Colon 26 Model, *Proc. Am. Assoc. Cancer Res.*, 38(3):305 (1997). This reference reported the HNTD of CPT-11 by i.v. administration to be 300 mg/kg. Moreover, the HNTD for intraperitoneal (i.p.) administration of CPT-11 in mice having colon tumors was determined to be 600 mg/kg. *See* Chatelut abstract.

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Gynecologic oncology, 76(3):362-8 (2000). This reference reported the HNTD for doxorubicin by i.v. on human ovarian cancer to be 16 mg/kg (8 mg/kg x 2).

After determining the HNTD for each constituent, Applicant combined the constituents to determine if there was a therapeutic synergistic effect, *i.e.*, whether smaller doses of each individual constituent could be used in a composition and yet still achieve a result better than the HNTD of one of the individual constituents when used alone. Applicant demonstrated such a therapeutic synergistic effect in several combinations reported in Table IV on page 11 of the specification.

Furuta does not teach the HNTD dose for each individual constituent. In fact, Furuta determined the dose for each constituent not based upon the HNTD for each constituent, but instead used amounts that would produce a desired result, around 150 of the life prolonging rate T/C (%). *See* page 394, first paragraph. For example, Furuta determined that a dose of 37.5 mg/kg (12.5 x 3 days of administration on days 1, 5, 9) of CPT-11 by i.p. administration in mice having L1210 (leukemia) tumors achieved that goal. *See* Table 3. (Applicant notes that this dose is $1/16^{th}$ of the reported HNTD for i.p. administration of CPT-11 in mice having colon cancer. *See* footnote 2.) Similarly, Furuta determined that a dose of 18.75 mg/kg (6.25 X 3 days of administration on days 1, 5, 9) of doxorubicin i.p. administered in mice having L1210 (leukemia) tumors achieved that goal.[2]

The Examiner previously argued that the data in Table 3 "shows that the effect of two chemicals on the inoculated mice is greater than the effect of each of these

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2 The HNTD for i.p. administration of doxorubicin in mice having lung carcinoma was determined to be 15 mg/kg. *See* Schmid, *et al.*, Differential Uptake of 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea and Doxorubicin by Lewis Lung Carcinoma and Ridgway Osteoganic Sarcoma, *Cancer-Res.*, 43(3):976-9 (1983).

chemicals individually" and refers to the example having 37.5 mg/kg CPT-11 (12.5 x 3 days of administration) and 18.75 mg/kg of adriamycin (6.25 x 3 days of administration) producing 16.5 days of survival as exhibiting a synergistic effect. *See* Office Action dated February 5, 2003, at 7 and Experiment 1 in Table A below. Applicant respectfully submits that the Examiner's statement is inconsistent with Applicant's definition of therapeutic synergistic effect.

Moreover, Applicant respectfully submits that the Examiner's statement is not supported by all, and is contrary to some, of the data in Furuta. For ease of understanding, Applicant refers to Table A below. The Examiner's statement that the effect of two chemicals is greater than the effect of each is true for experiment 1, wherein the days of survival are greater when two chemicals are used than the effect of each of these chemicals used individually (experiments 2 and 3). However, the Examiner's statement is contrary to the data in experiment 4 when it is compared to the data in experiment 3. In experiment 4, both chemicals are used and the days of survival is 11.7. In experiment 3, only doxorubicin is used and the days of survival are 11.7. The data in experiments 3 and 4 rebut the Examiner's statement that the effect of two chemicals is greater than the effect of each of these chemicals used individually. The data does not appear to suggest a therapeutic synergistic effect, as defined by the Examiner or, for that matter, the Applicant. Applicant notes that the Examiner has failed to respond to this point and requests his comments.

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Table A. Compilation of Data from Four Experiments in Furuta

Experiment	Total Dose of CPT-11 (12.5 mg/kg x 3 days of administration at days 1, 5, 9)	Total Dose of adriamycin (6.25 mg/kg x n days of administration)	Days of Survival
1	37.5	18.75, n=3	16.5
2	37.5	0	10.8
3	0	18.75, n=3	11.7
4	37.5	6.25, n=1	11.7

As further evidence that Furuta does not teach a therapeutic synergistic combination, Applicant refers the Examiner to the rate of survival of the mice in Furuta. Applicant notes that in experiments 2 and 3 in Table A above, the mice treated with one constituent survived about 12 days before they expired. In experiment 1 in Table A, the example relied upon by the Examiner as evidencing a therapeutic synergistic effect, the mice survived about 5 days longer for a total of 17 days.

In comparison, in the examples provided in the present specification in Table IV, the mice treated with CPT-11 alone survived for at least 26 days. (The data in Table IV is not directed to days of survival as in Furuta, but is instead directed to the time in days for the tumors to reach 1000 mg. Therefore, presumably, the mice lived longer than is reported.) The mice treated with doxorubicin alone survived for least 24 days. The mice that were treated with a therapeutic synergistic combination of CPT-11 and doxorubicin survived for at least 52 days. The rate of survival for mice treated with the therapeutic synergistic combination is almost double the rate of survival for the mice treated with the individual constituents alone, and more than three times the rate

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reported in Furuta. Applicants note that the Examiner failed to respond to this aspect of the response and requests his comments.

As explained above, Furuta fails to determine the HNTD for CPT-11 and adriamycin, and therefore one of ordinary skill in the art cannot conclude that the results reported by Furuta demonstrate a therapeutic synergistic effect as defined by the presently claimed invention. Moreover, Furuta conducts all of his experiments on leukemia cells in mice. Furuta does not teach a composition that provides a therapeutic synergistic effect in the treatment of solid tumors, as presently claimed. Success in treating leukemia cannot be equated to success in treating solid tumors for reasons elucidated below. For at least these reasons, Furuta does not anticipate the claimed invention.

REJECTION UNDER 35 U.S.C. § 103(a)

The Examiner also maintains the rejection of claims 5, 8, and 13-20 under 35 U.S.C. § 103(a) over Furuta.[3] The Examiner restates his rejection on pages 3-5 of the present Office Action.

With regard to claims 5 and 7, the Examiner argues that Furuta teaches adriamycin and etoposide, which are both well known anthracycline antibiotics or antitumor agents of very similar structure. Office Action at 3. With regard to claim 13, the Examiner argues that the "difference between applicant's claimed method and the method taught by Furuta et al. is that the applicant's type of tumor that is treated." *Id.* at

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3 Applicant notes that in the discussion of this particular rejection, the Examiner refers to claim 7 when discussing epipodophyllotoxin teniposide. Office Action at 3. In order to clarify the record, epipodophyllotoxin etoposide is recited in claim 7 and epipodophyllotoxin teniposide is recited in claim 8, which is listed in the formal rejection.

5. Because Furuta teaches a method for treating a leukemia tumor, the Examiner argues that it would have been obvious to treat other tumors, such as solid tumors, using the antitumor agents taught in Furuta "based on need, like the type and/or degree of severity of the tumor." *Id.*

To establish a *prima facie* case of obviousness, the reference must teach or suggest all the claim elements or must provide some suggestion or motivation to one of ordinary skill in the art to modify the reference. Additionally, there must be a reasonable expectation of success. M.P.E.P. § 2143 (8th ed., 2001). Applicant respectfully traverses the rejection and submits that Furuta would not have rendered obvious the claimed invention.

Claims 5 and 7 (or 8) indirectly depend from independent claim 1 and are therefore patentable for the same reasons as claim 1. Because, as discussed above, Furuta does not teach determining the HNTD of either CPT-11 or adriamycin alone, or finding a combination that provides a therapeutic synergistic effect that is superior to one of these doses, Furuta does not suggest a therapeutic synergistic composition according to the present claims. For at least this reason, Furuta fails to render the presently claimed invention obvious.

Furthermore, in rejecting the claims under 35 U.S.C. § 103(a), the Examiner fails to indicate where or how Furuta provides a reasonable expectation of success in achieving a therapeutic synergistic composition that is useful in treating solid tumors. Furuta discloses treatment of leukemia. Furuta does not disclose or suggest treating solid tumors with CPT-11 and adriamycin. The Examiner minimizes this omission, asserting that one would apply the teachings of Furuta to any type of tumor. Applicant respectfully disagrees.

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It is widely recognized in the art of tumor therapy that a treatment regimen that is successful against one type of tumor (*e.g.*, leukemia) will not necessarily be successful against other types of tumors (*e.g.*, solid tumors). For example, L. M. Van Putten teaches that "[i]t is possible that differences in cellular biochemistry may be responsible for explaining the difficulty of treating by means of chemotherapy the majority of solid tumors [as compared to leukemias]." *See* Abstract of L. M. Van Putten, "Recruitment, a double-edged sword in cancer chemotherapy," *Bulletin du Cancer*, 60(2):140-141 (1973), a copy is attached for the Examiner's convenience as Exhibit A. Moreover, it has been noted that "solid tumors are less sensitive to apoptosis induced by anticancer drugs than leukemias and lymphomas." *See* Abstract of M. Kawada, "Development of a Selective Apoptosis Inducer of Solid Tumor Cells," *Biotherapy*, 12(6):967-973 (1998), attached as Exhibit B.

Thus, even assuming for the sake of argument that one were to be motivated by Furuta to apply the teachings of Furuta to other cancers, such as solid tumors, that person would have no reasonable expectation of success in treating the solid tumors. This is especially so because Furuta does not teach or suggest how to determine the HNTD nor how to use that information to arrive at the most efficacious combination dose of the treatment compounds.

Rather, at most, one of skill in the art would see Furuta as a mere invitation to attempt to treat solid tumors, not a guarantee of success. In other words, any motivation that Furuta might provide would be a motivation to try, not a motivation to succeed. M.P.E.P. § 2145 X.B. prohibits rejections under this theory. Indeed, it is only through the teachings of the present specification that one of ordinary skill in the art would gain a reasonable expectation of success in treating solid tumors with a

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therapeutic synergistic combination of camptothecin, or a camptothecin derivative, and a topoisomerase II inhibitor. However, Applicant's own disclosure cannot provide the motivation or expectation of success necessary to render a claim obvious.

Therefore, because Furuta does not provide an adequate motivation or reasonable expectation of achieving the presently claimed invention, Applicant respectfully submits that the presently claimed invention would not be obvious over Furuta.

CONCLUSION

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration of the application, and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
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Dated: December 3, 2003

By: Carol L. Cole

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Attachments:
Exhibit A
Exhibit B

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BULLETIN DU CANCER

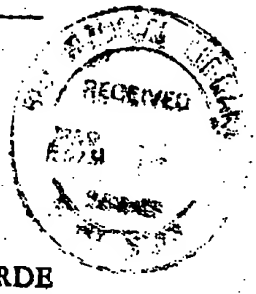

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Exhibit A

(9) GRISWOLD (D. P.), SCHABEL (F. M.), WILCOX (W. S.), SIMPSON HERREN (L.) and SKIPPER (H. E.). — Success and failure in the treatment of solid tumors. *Cancer Chemother. Rep.*, 1968, 52, 345-387.
(10) GABUTTI (V.), PILERI (A.), TAROCCO (R. P.), GAVOSTO (F.) and COOPER (E. H.). — Proliferative potential of out-of-cycle leukaemia cells. *Nature (Lond.)*, 1969, 224, 375-376.
(11) LAMPKIN (B. C.), NAGAO (T.) and MAUER (A. M.). — Synchronization and recruitment in acute leukemia. *J. clin. Invest.*, 1971, 50. 2204-2214.
(12) BRUCE (W. R.), MEEKER (B. E.), POWERS (W .E.) and VALERIOTE (F. A.). — Comparison of the dose— and time— survival curves for normal hematopoietic and lymphoma colony-forming cells exposed to Vinblastine, Vincristine, Arabinosylcytosine and Amethopterin, *J. nat. Cancer Inst*, 1969, 42, 1015-1025.
(13) HANKS (G. E.). — Recruitment and altered recovery in noncycling bone marrow stem cells. *Radiology*, 1972, 103, 691-693.
(14) BRUCE (D. L.). LIN (H. S.) and BRUCE (W. R.). — Reduction of colony-forming cell sensitivity to arabinosylcytosine by halothane anaesthesia. *Cancer Res.*, 1970, 30, 1803-1805.
(15) BRUCE (W. R.), MEEKER (B. E.) and VALERIOTE (F. A.). — Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells tochemotherapeutic agents administered *in vivo*. *J. nat. Cancer Inst.*, 1966, 37, 233-245.
(16) BRUCE (W. R.) and VALERIOTE (F. A.). — Normal and malignant Stem cells and chemotherapy. *In: The proliferation and spread of Neoplastic cells*, (Williams and Wilkins, C°, édit.), Baltimore, 1968, 409-422.
(17) PUTTEN (L. M. van) and LELIEVELD (P.). — Factors determining cell killing by chemotherapeutic agents *in vivo*. I. *Europ. J. Cancer*, 1970, 6, 313-321.
(18) WODINSKY (I.), SWINIARSKI (J.) and KENSLER (C. J.). — Spleen colony studies of leukemia L 1210. II Differential sensitivities of normal and leukemic bone marrow colony-forming cells to single and divided dose therapy with cytosine arabinoside. *Cancer Chemother. Rep.*, 1967, 51, 423-429.
(19) VALERIOTE (F. A.) and BRUCE (W. R.). — Comparison of the sensitivity of hematopoietic colony-forming cells in different proliferative states to Vinblastine. *J. nat. Cancer Inst.*, 1967, 38, 393-399.
(20) TWENTYMAN (P. R.) and BLACKETT (N. M.). — Action of cytotoxic agents on the erythroid system of the mouse. *J. nat. Cancer Inst.*, 1970, 44, 117-123.
(21) SKIPPER (H. E.), SCHABEL (F. M. Jr) and WILCOX (W. S.). — Experimental evaluation of potential anti-cancer agents XIII. On the criteria and kinetics associated with « curability » of experimental leukaemia. *Cancer Chemother. Rep.*, 1964, 35, 1-111.
(22) BERGSAGEL (D. E.) and VALERIOTE (F. A.). — Growth characteristics of a mouse plasma cell tumor. *Cancer Res.*, 1968, 28, 2187-2196.
(23) HERMENS (A. F.) and COOLEN (J.). — Unpublished observations, 1973.

SUMMARY

Recruitment, a double-edged sword in cancer chemotherapy

by L. M. VAN PUTTEN

Work done with drugs which act on a phase of the cell cycle has shown that the substances which most rapidly reduce the volume of experimental tumours are equally those which are the most effective in provoking recruitment of haemopoietic stem cells (colony forming units in the spleen). The recruitment of quiescent cells may equally sensitise the normal tissues with regard to the toxic effect of the drugs. Besides, we have shown an important difference between the sensitivity to antimetabolites of leukaemia L1210 and a solid transplantable tumour,

while nevertheless in both of the systems the cells are in rapid proliferation. This observation suggests that the generally lower sensitivity of solid tumours to cytostatic agents is not necessarily related to differences in cell kinetics.

It is possible that differences in cellular biochemistry may be responsible for explaining the difficulty of treating by means of chemotherapy the majority of solid tumours.

RESUMEN

Reclutamiento, un arma de dos filos en la quimioterapia del cáncer

por L. M. Van Putten

Los trabajos realizados con las drogas que actúan sobre una fase del ciclo han mostrado que las sustancias que reducen más netamente el volumen de los tumores experimentales son igualmente aquellas que son más eficaces para provocar un reclutamiento de las células cepas hematopoyéticas (células formadoras de colonias en el bazo). Este fenómeno del reclutamiento de las células « quiescentes » puede igualmente sensibilizar los tejidos normales respecto al efecto tóxico de los medicamentos. Además, los autores han puesto en evidencia una diferencia importante entre la sensibilidad a los antimetabólicos de la leucemia L1210 y de un tumor sólido injertable, no obstante en los dos sistemas todas las células están en proliferación rápida. Esta observación sugiere que la sensibilidad generalmente más baja de los tumores sólidos a los agentes citostáticos no está necesariamente en relación con diferencias de cinética celular.

Es posible que las diferencias de bioquimia celular estén en causa para explicar la dificultad de tratar por quimioterapia la mayor parte de los tumores sólidos.

特集

がん化学療法の新しいターゲット—アポトーシスを中心に—

Ⅲ．アポトーシスをターゲットとした新規抗がん剤のスクリーニング

固形癌に有効なアポトーシス誘導物質を求めて

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要旨 一般的に固形癌細胞は血球系の癌細胞よりも抗癌剤によりアポトーシスを起こしにくい。固形癌により有効にアポトーシスを誘導する経路が存在すれば，それを新物質の探索系として応用することが可能であろう。Bactobolin は B16 メラノーマ細胞に，Cytostatin は EL-4T リンパ腫細胞に選択的にアポトーシスを誘導した。これらのアポトーシス誘導では CPP32 が活性化したが，CPP32 の阻害剤は B16 細胞のアポトーシスのみを抑制した。また Bactobolin によって B16 細胞のすべての cyclin が減少したが，EL-4 細胞では cyclin A と B が減少しなかった。一方 Cytostatin によって EL-4 細胞のすべての cyclin が減少したが，B16 細胞では cyclin E のみが減少しなかった。これらの結果は両細胞間でのアポトーシス誘導機構の違いを反映していると考えられる。

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Development of a Selective Apoptosis Inducer of Solid Tumor Cells

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Summary

Most solid tumor cells are less sensitive to apoptosis induced by anticancer drugs than leukemias and lymphomas. A selective apoptotic pathway of solid tumor cells may possibly be useful to develop a new strategy for screening of apoptosis inducers. Bactobolin selectively induced apoptosis of B16 melanoma cells and Cytostatin selectively did so in EL-4 T lymphoma cells. While CPP32 was activated on the apoptosis of both cell lines, an inhibitor of CPP32 suppressed only the apoptosis of B16 cells. Bactobolin decreased all cyclins in apoptotic B16 cells, but not cyclin A and B in EL-4 cells. On the other hand, Cytostatin decreased all cyclins in apoptotic EL-4 cells, but not cyclin E in B16 cells. These results indicated that the apoptotic pathways were different between both cell lines.

Key words: Solid tumor, Apoptosis, Bactobolin, Cytostatin, Cell cycle

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Exhibit B

はじめに

一細胞単位で起こる積極的な細胞死であるアポトーシスは，もともと生体の形態形成において不要な細胞を除去するメカニズムとして注目された分野であるが，現在では多くの癌研究者にとって癌の発生あるいは治療といった観点から主要な癌研究分野となっている。それは，癌が本来死ぬべきはずの細胞が不死化した細胞，つまりアポトーシスの異常な細胞集団であるといった見方ができるからであろう。さらには，これまで臨床で使用されている抗癌剤の多くが癌細胞にアポトーシスを誘導するという事実から[1-4]，新しい抗癌剤のターゲットとしてますます注目されるようになった。しかしながら抗癌剤の癌細胞への作用を比較すると，一般的に白血病など血球系由来の癌細胞のほうが固形癌由来の癌細胞よりも抗癌剤によるアポトーシス誘導に高感受性である傾向がみられる。これは臨床における固形癌の抗癌剤に対する低感受性を半ば反映しているようにも思える。固形癌に対する癌化学療法の向上をめざす上で，アポトーシスを抗癌剤のターゲットとするならば，固形癌により有効にアポトーシスを誘導する薬剤の発見が望まれる。固形癌に選択的にアポトーシスを誘導する経路（ターゲット）が存在すれば，それを新物質の探索系として応用することが可能であろう。しかし，近年アポトーシスにかかわる様々な因子が発見されているが，このような血球系と固形癌由来の癌細胞間での感受性の差がなにに起因するのかはよくわかっていない。そこで，新たな探索系を開発する目的で両細胞系を用いて薬剤によるアポトーシス誘導機構の違いを解析した。

I. 抗癌剤によるアポトーシス

固形癌と血球系の癌細胞間でのアポトーシス感受性の差を解析するために，比較的アポトーシスが誘導されにくい細胞をモデル系として選択することにした。まず固形癌由来の癌細胞としてマウスB16メラノーマ細胞を，また血球系由来としてマウスEL-4 Tリンパ腫細胞を用いたモデル系について検討した。アポトーシス研究でよく使用されるヒトHL-60細胞と比較して，両細胞とも薬剤などの処理により比較的アポトーシスが誘導されにくい細胞である（未発表）。表1に作用機序の異なる代表的な抗癌剤による両細胞のアポトーシス感受性を示した。アポトーシスの誘導は，24時間細胞を薬剤で処理した後にDNAの断片化を指標にした。その結果，ほとんどの抗癌剤が両細胞にアポトーシスを誘導することがわかったが，両細胞の感受性に着目すると，やはり血球系由来のEL-4細胞のほうが固形癌由来のB16細胞よりも同じ処理時間においてアポトーシスが誘導されやすいことがわかった。またB16細胞に対して強くアポトーシスを誘導する物質も用いた抗癌剤のなかにはみられなかった。このようにこのモデル系はわれわれが着目している抗癌剤によるアポトーシスの問題点をよく反映していると考えられる。そこで，このモデル系を用いていくつかの薬剤についてアポトーシス誘導能を検討した。

II. CytostatinおよびBactobolinによるアポトーシス

Bactobolinは1979年に抗菌および抗白血病作用を有する抗生物質として微生物化学研究所で発見された（図1）[5,6]。Cytostatinは B16細胞の細胞外マトリックスへの接着を阻害する物質として放線菌より得られた新規物質である（図1）[7-9]。これらの物質はどちらも別々にアポトーシス誘導

表1 主な抗癌剤によるアポトーシス感受性

	B16	EL-4
Methotrexate		+
5-FU		+
Adriamycin	+++	+++
Aclacinomycin	+	++
Bleomycin		+
Mitomycin C	+	+
Actinomycin D	+	++
Vinblastine	+	++
Vincristine	+	+++
Taxol	+	+
Etoposide	+	+++
Camptothecin		++
Cisplatin	+	++

薬剤で細胞を24時間処理後，断片化されたDNAを定量した。表には各薬剤のDNA断片化誘導能を相対的に表した。

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図1)[7-9]。
シス誘導

感受性

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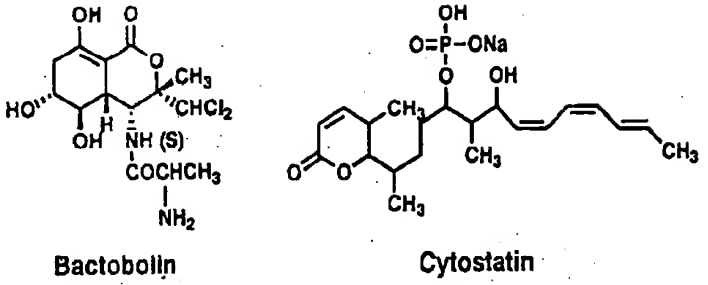

図 1 Bactobolin と Cytostatin

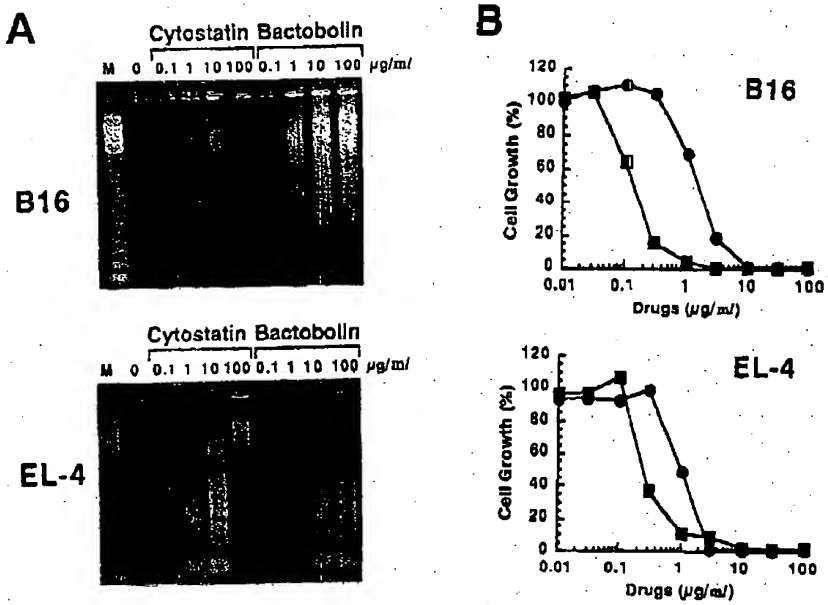

図 2 Bactobolin, Cytostatin によるアポトーシス誘導と増殖阻害
A：薬剤処理後 B16 細胞は 24 時間，EL-4 細胞は 6 時間後に断片化した DNA をアガロースゲル電気泳動で検出した．M，マーカー。
B：薬剤処理 3 日後の細胞増殖阻害を MTT を用いて調べた。
●，Cytostatin；■，Bactobolin。

能が認められていたが[10]，本研究で用いたモデル系において興味深い結果が得られた。B16 および EL-4 細胞に対するアポトーシス誘導能を比較したところ，B16 細胞では Bactobolin が Cytostatin よりも低濃度の 1 μg/ml から 24 時間処理で強く DNA 断片化を引き起こしたのに対して，EL-4 細胞では Cytostatin が Bactobolin よりも低濃度の 1 μg/ml から 6 時間処理で強く DNA 断片化を引き起こした（図 2A）。しかしこれら薬剤による細胞増殖阻害を比較すると B16 細胞では IC_{50} にして Bactobolin が 0.2 μg/ml，Cytostatin が 2 μg/ml に対して，EL-4 細胞では Bactobolin が 0.2 μg/ml，Cytostatin が 1 μg/ml であり，どちらの細胞も Bactobolin が Cytostatin よりも低濃度で増殖を阻害した（図 2B）。この増殖阻害の結果はアポトーシス誘導と相関しないことから，アポトーシス誘導の差は単なる薬剤感受性の違いではないと考えられる。このように Bactobolin は

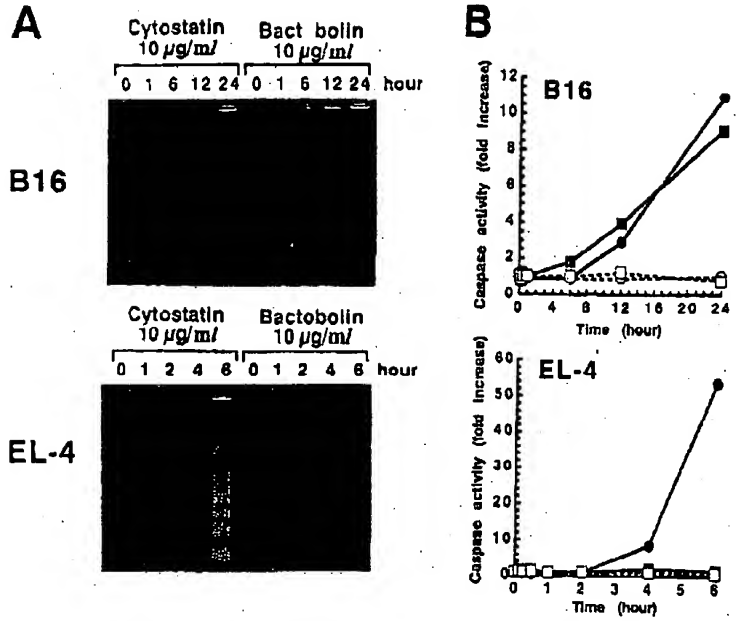

図 3 Bactobolin, Cytostatin による Caspase ファミリーの活性化
A : Bactobolin, Cytostatin による DNA 断片化を経時的に観察した。
B : ICE (○, □) と CPP32 (●, ■) の活性を経時的に測定した。
○, ●. Cytostatin ; □, ■, Bactobolin。

B16 細胞に Cytostatin は EL-4 細胞に選択的にアポトーシスを誘導することがわかった。これらの結果は固形癌と血球系癌とのアポトーシス感受性の差の解析に有用であると考えられたので，両薬剤と細胞のモデル系を用いてさらに解析を行った。

III. Caspase 活性とアポトーシス

アポトーシスにおいて Caspase ファミリーの活性化が重要な役割を担っていることが知られている。たとえば生理的なものとしては，Fas などを介したアポトーシスでは ICE (Caspase-1) および CPP32 (Caspase-3) が順次活性化する[11]。一方，抗癌剤などによるアポトーシスでも CPP32 が活性化することが知られている[12, 13]。そこで，Bactobolin および Cytostatin によるアポトーシスにおける Caspase ファミリーの関与について検討した。ICE および CPP32 の活性は特異的な蛍光基質 Ac-YVAD-MCA および Ac-DEVD-MCA をそれぞれ用いた。B16 細胞では Bactobolin が Cytostatin よりも強くアポトーシスを誘導するが，どちらの処理によっても CPP32 は経時的に同程度活性化し，DNA 断片化と相関しなかった（図 3）。一方，EL-4 細胞ではアポトーシス誘導の強い Cytostatin によって CPP32 が活性化したが，アポトーシス誘導の弱い Bactobolin では活性化しなかった（図 3）。またどちらの細胞においても ICE は活性化しなかった（図 3）。次に実際に Caspase の阻害でアポトーシスが抑制できるのか検討した。ICE および CPP32 の阻害剤としては Ac-YVAD-CHO (YVAD) および Ac-DEVD-CHO (DEVD) をそれぞれ用いた。B16 細胞での Bactobolin によるアポトーシスは DEVD によって抑制されたが，YVAD では抑制されなかった（図 4）。また Cytostatin も弱くアポトーシスを誘導するがこれも DEVD で抑制された。一方，EL-4 細胞の Cytostatin によるアポトーシスは DEVD, YVAD どちらでも抑制されなかった（図 4）。したがって B16 細胞に対して Bactobolin は CPP32 を活性化することでアポトーシスを誘導するが，Cytostatin は CPP32 以降のシグナルが不十分で

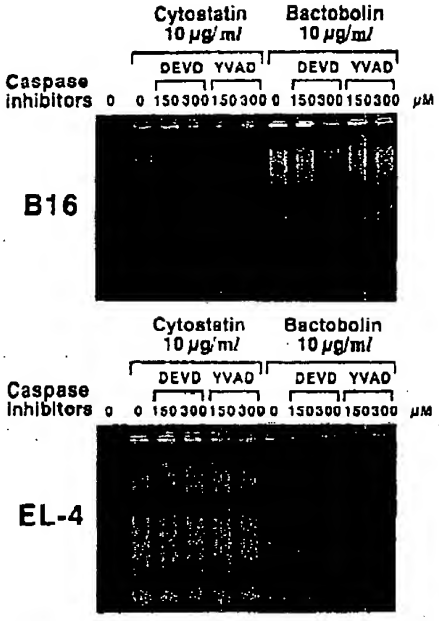

図4 Bactobolin, Cytostatinによって誘導されたアポトーシスに対するCaspase阻害剤の影響
薬剤処理後にB16細胞は24時間，EL-4細胞は6時間後にDNA断片化を検出した。DEVDはCPP32のYVADはICEの阻害剤。

あるためアポトーシス誘導が弱いと考えられる。一方，EL-4細胞に対してCytostatinはCPP32を活性化したが，CPP32の阻害剤でアポトーシスが抑制されないことから，別の経路を介してアポトーシスを誘導している可能性が考えられ，さらにBactobolinはこのシグナルが十分でないと予想された。これらの結果はCPP32の活性化が必ずしもアポトーシスと相関しないことを示しているといえる。またEL-4細胞でCPP32の活性化がB16細胞よりも非常に高くアポトーシス感受性との関係が興味深いが，われわれの結果からどうやら直接的な関係はないのかもしれない。

IV. 細胞周期とアポトーシス

アポトーシスにかかわる因子として，アポトーシスの誘導に働くBaxや抑制に働くBcl-2などの蛋白因子が知られている[14, 15]。また細胞周期との関係では，増殖または死を選択するチェックポイントの存在が示唆されている。そこでこれら蛋白因子に対するBactobolinおよびCytostatinの影響を検討した。

1. アポトーシス関連因子

B16細胞ではアポトーシス誘導の強いBactobolinおよび弱いCytostatinいずれもBcl-2が減少し，続いてBcl-xおよびBaxが減少した(図5)。一方，EL-4細胞ではBactobolinあるいはCytostatinによってBaxおよびBcl-2はまっ

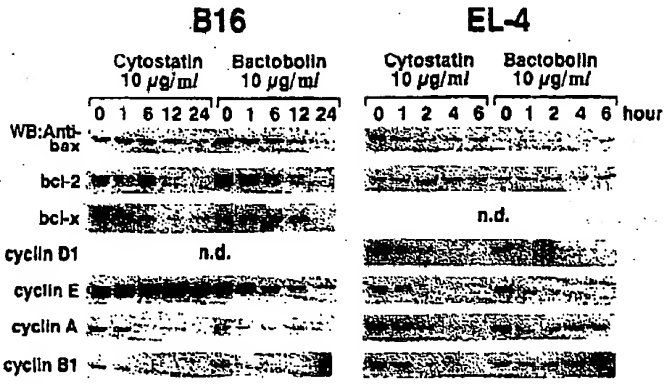

図5 Bactobolin, Cytostatinのアポトーシスおよび細胞周期関連因子への影響
薬剤を各時間で処理後，細胞内蛋白を抽出し，SDSポリアクリルアミドゲル電気泳動後，各蛋白因子をウエスタンブロッティング法にて検出した。
n.d., 検出限度以下。

たく変化しなかった（図5）。これらの結果からB16細胞ではアポトーシス抑制因子のBcl-2の減少とCPP32の活性化に相関がみられ，アポトーシスとの関連が示唆された。一方，EL-4細胞ではBcl-2の減少とCPP32の活性化の間に相関はなく，B16細胞とは基本的に異なるメカニズムが存在していると考えられた。

2. 細胞周期関連因子

B16細胞ではアポトーシスを強く誘導するBactobolinよって，cyclin E，A，B，すべてのcyclinが減少したが，アポトーシス誘導の弱いCytostatinではcyclin AとBは素早く減少するが，cyclin Eが減少しないことがわかった（図5）。一方，EL-4細胞ではアポトーシスを強く誘導するCytostatinではすべてのcyclinが減少したが，アポトーシス誘導の弱いBactobolinではcyclin A，Bが減少しなかった（図5）。これらの結果から，アポトーシスの条件においてはそれに先行してすべてのcyclinの減少を伴うと考えられる。またB16細胞ではcyclin E，EL-4細胞ではcyclin AとBが残っているとアポトーシスが起きないことから，それぞれのcyclinがアポトーシスに対して抑制的に働いている可能性が考えられた。また，それらのcyclinが関与する細胞周期の位置がアポトーシスのチェックポイントとして働いているのかもしれない。

おわりに

これまでに使用されている抗癌剤は確かに実験レベルでは細胞にアポトーシスを誘導するが，実際に臨床においてアポトーシスを実質的に誘導しているのかはよくわからない点である。また癌治療という面からもネクローシスではなくてアポトーシスを誘導させることが治療向上につながるのかという点についても答えがでていないと思う。そのためにもアポトーシス誘導物質の意義は大きいように考えられる。しかしながらアポトーシス誘導物質はいわゆる細胞毒性物質という側面ももっているわけであるので，なんらかの選択性，たとえば細胞内シグナルや細胞外のイベントまたは臓器特異性をもったものでなくてはならないように思う。本研究では固形癌と血球系との差に注目したが，それぞれの細胞間でのアポトーシスの誘導機構が明らかに異なることを示せたように思う。実際の違いについては他の多くの細胞について行う必要があるが，cyclinなどを選択的に操作できればアポトーシスをコントロールできる可能性が考えられる。今後，この結果を新物質の探索系へ役立てられればと考える。

文献

1) Yoshida, A., Ueda, T., Wano, Y. *et al.*: DNA damage and cell killing by camptothecin and its derivative in human leukemia HL-60 cells. *Jpn. J. Cancer Res.* 84 : 566-573, 1993.
2) Ling, Y., Priebe, W. and Perez-Soler, R.: Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. *Cancer Res.* 53 : 1845-1852, 1993.
3) Evans, D. and Dive, C.: Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. *Cancer Res.* 53 : 2133-2139, 1993.
4) Haldar, S., Chintapalli, J. and Croce, C.: Taxol induces *bcl-2* phosphorylation and death of prostate cancer cells. *Cancer Res.* 56 : 1253-1255, 1996.
5) Kondo, S., Horiuchi, Y., Hamada, M. *et al.*: A new antitumor antibiotic, bactobolin produced by *Pseudomonas*. *J. Antibiotics* 32 : 1069-1071, 1979.
6) Ishizuka, M., Fukasawa, S., Masuda, T. *et al.*: Antitumor or effect of bactobolin and its influence on mouse immune system and hematopoietic cells. *J. Antibiotics* 33 : 1054-1062, 1980.
7) Amemiya, M., Uono, M., Osono, M. *et al.*: Cytostatin, a novel inhibitor of cell adhesion to components of extracellular matrix produced by *Streptomyces sp.* MJ654-NF4. *J. Antibiotics* 47 : 536-540, 1994.
8) Amemiya, M., Someno, T., Sawa, R. *et al.*: Cytostatin, a novel inhibitor of cell adhesion to components of extracellular matrix produced by *Streptomyces sp.* MJ654-NF4. *J. Antibiotics* 47 : 541-544, 1994.
9) Masuda, T., Watanabe, S., Amemiya, M. *et al.*: Inhibitory effect of cytostatin on spontaneous lung metastases of B16-BL6 melanoma cells. *J. Antibiotics* 48 : 528-529, 1995.
10) Yamazaki, K., Amemiya, M., Ishizuka, M. *et al.*: Screening for apoptosis inducers in microbial products and induction of apoptosis by cytostatin. *J. Antibiotics* 48 : 1138-1140, 1995.
11) Enari, M., Talanian, R., Wong, W. *et al.*: Sequential activation of ICE-like and CPP32-

like proteases during Fas-mediated apoptosis. *Nature* 380 : 723-726, 1996.
12) Nicholson, D., Ali, A., Thornberry, N. *et al.* : Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376 : 37-43, 1995.
13) Chen, Z., Naito, M., Mashima, T. *et al.* : Activation of actin-cleavable interleukin 1β-converting enzyme (ICE) family protease CPP-32 during chemotherapeutic agent-induced apoptosis in ovarian carcinoma cells. *Cancer Res.* 56 : 5224-5229, 1996.
14) Vaux, D., Cory, S. and Adams, J. : *Bcl-2* gene promotes haemopoietic cell survival and cooperates c-*myc* to immortalize pre-B cells. *Nature* 335 : 440-442, 1988.
15) Oltvai, Z., Milliman, C. and Korsmeyer, S. : Bcl-2 heterodimerizes *in vivo* with a conserved homolog, bax, that accclerates programmed cell death. *Cell* 74 : 609-619, 1993.